

Seminiferous tubule number and surface: validation of objective parameters to estimate reproduction activity of male European anchovy (*Engraulis encrasicolus*, L.)

by

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Abstract. – The present study aims to establish new quantitative parameters to objectively study reproductive activity in the male anchovy, *Engraulis encrasicolus*. The experimental design measured, at a microscopic level, the number (STN) and area (STA) of the seminiferous tubules during the reproductive season. This was conducted in relation to gonadosomatic index (GSI) and the five-degree macroscopic standard maturity staging of Brown-Petersen *et al.* (2011): immature, developing, spawning capable, regressing, regenerating. Fresh samples with a total of 200 fish were collected weekly during the reproduction season from October 2007 to May 2008 from commercial catches in the Gulf of Bejaia (Northeast of Algeria). Spermatogenesis activity is recorded by STN and STA values allowing the five Brown-Petersen stages to be distinguished and STN and STA also revealed accurately the different reproduction events during the study period (from October to May). In conclusion, the current results demonstrate that standardized STN and STA allow precise quantitative study of the reproduction activity of the male anchovy, and possibly in other fish species. The present method, based exclusively on histological slides biometry, could be recommended as a standard technique.

Résumé. – Nombre et surface des tubules séminifères : validation de paramètres objectifs pour l'étude de l'activité de reproduction de l'anchois mâle (*Engraulis encrasicolus*, L.).

L'objectif de la présente étude était d'explorer l'intérêt de deux nouveaux paramètres biométriques histologiques au niveau testiculaire pour l'étude objective de la dynamique de reproduction des mâles d'anchois, *Engraulis encrasicolus*. Les informations apportées par deux paramètres biométriques mesurés à l'échelle microscopique, le nombre de tubules séminifères (STN) et leur surface (STA) sont comparées au rapport gonadosomatique (RGS) et au stade de maturité sexuelle dans l'échelle de Brown-Petersen *et al.* (2011). Un échantillonnage hebdomadaire, avec un effectif total de 200 individus, a été effectué pendant la saison de reproduction d'octobre 2007 à mai 2008 dans des captures commerciales dans le golfe de Bejaia (nord-est de l'Algérie). Une similitude a été observée entre le RGS et les stades de maturité d'une part et les valeurs de STN et STA d'autre part. Ces deux dernières ont évolué de façon inverse l'une par rapport à l'autre, au moment où la surface des tubules séminifères augmente leur nombre diminue, indiquant ainsi une activité de spermatogenèse avec une activité mitotique intense. Ce qui fait que les cinq étapes de Brown-Petersen peuvent être discriminées en utilisant les deux index, mieux qu'en utilisant uniquement RGS. La dynamique de reproduction du mâle de l'anchois est décrite par les deux paramètres biométriques (STN et STA). En conclusion, ils pourraient être utilisés comme un outil fiable pour l'étude quantifiée de l'activité de reproduction des mâles d'anchois, et probablement d'autres poissons. Cette approche, basée sur de la biométrie sur des tissus en coupe offre une alternative intéressante dans la standardisation des études de la reproduction.

The European anchovy, *Engraulis encrasicolus* (Linnaeus, 1758), has a widespread distribution. This fish species lives on the eastern Atlantic coasts, from Scandinavia to Western Africa (Ba, 1988). In the Mediterranean Sea, the most important locations for this resource are the Balearic Sea, the Gulf of Lions, the western Mediterranean Sea, the Adriatic and the Aegean seas (Mazzola *et al.*, 2002). Studies of the reproduction of *E. encrasicolus* in both Mediterranean and Atlantic waters assessed that the breeding season runs from April to November, with a peak activity occurring from the middle of June to the end of July (Giraldez and Abad, 1995; Motos *et al.*, 1996; Millan, 1999; Palomera *et al.*, 2003; Gaamour *et al.*, 2004; Basilone *et al.*, 2006). The

economic importance of this resource has significantly influenced a large number of academic research studies within each region, especially in relation to stock assessment, reproductive traits and biology of this species (Garcia and Palomera, 1996).

Concerning the reproduction biology, most published works are limited to macroscopic evaluation of the maturity stage, including the GSI (Sanz and Uriarte, 1989; Palomera, 1992; Gaamour *et al.*, 2004) and its validation (Rampa *et al.*, 2005; Ferreri *et al.*, 2009). For instance, the Daily Egg Production Method (DEPM) was developed in the late 1970's by the Coastal Division of the Southwest Fisheries Center, La Jolla, CA, USA (Parker, 1980) as a response to a grow-

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ing need for a suitable and direct method for reproduction activity assessment. However, these macroscopic analyses do not lead to sufficiently detailed and accurate recordings to prevent discrepancies between laboratories (Ferreri *et al.*, 2009).

In contrast, histological analysis is revealed to be a valuable technique resulting in a accurate determination of fish reproduction activity (Monsefi *et al.*, 2009). Traditionally, different fish species have been subject to histological techniques, which essentially focused on females, by providing information on ovary structure, cell organization and activity along the sexual maturation process (Rampa *et al.*, 2005; Ferreri *et al.*, 2009). Biometric parameters, such as oocyte diameter, are also reported as objective in determining the annual breeding cycle and gonads development (Kaneko and Hanyu, 1985; La Mesa *et al.*, 2003; Barone *et al.*, 2007; Berra *et al.*, 2007; Monsefi *et al.*, 2009). In this respect, positive correlations have been observed between microscopic biometric values, GSI and maturity stages (Chellappa *et al.*, 1989; Valdés *et al.*, 2004; Vallisneri *et al.*, 2006; Kanak and Tachihara, 2008; Shalloof and Salama, 2008; Dorostghoal *et al.*, 2009; Lawson and Aguda, 2010; Muchlisin *et al.*, 2010; Lawson, 2011).

With regards to male fish, only a few quantitative analyses have been dedicated to investigating spermatogenesis activity and its relationship with factors related to the individual or environmental variations. The common approach is based on counting the number of germ cells per tubule section; however, this remains lengthy and complex, because of the small-size of sex cells in males (Billard *et al.*, 1974; Muñoz *et al.*, 2002).

Male anchovy display anastomosing seminiferous tubules (Parenti and Grier, 2004). Despite all published works, little is however reported regarding their gonad histology, and much concerns the description of spermatic cells, relating to the sexual maturity stages (Hemida, 1987; Melo, 1994; Vallisneri and Scapolatempo, 2000; Rampa *et al.*, 2005; Manzo *et al.*, 2013). Among other clupeiform fishes, male gonad histology is also limited to qualitative observations (Landry and McQuinn, 1988; Bucholtz *et al.*, 2008; Pešić *et al.*, 2010; Osman *et al.*, 2011; Coterio-Altamirano *et al.*, 2015).

To the best of our knowledge, no report has been published regarding the histological quantification of male anchovy gonad activity and its relationships with current macroscopic parameters. The present work investigates the relationship of two biometric parameters, the number (STN) and the area (STA) of anchovy seminiferous tubules, as indicators of the reproduction activity. The experimental design aims at exploring the relationships between these two microscopic parameters and the conventional reproductive indexes, particularly GSI and anchovy maturity stages.

MATERIAL AND METHODS

Fish sampling and laboratory protocols

Random samples of *Engraulis encrasicolus* were collected from the commercial landings of the purse-seine fleet at the fishing port of Bejaia (Northeast of Algeria) on a weekly basis from October 2007 to May 2008, which covers the anchovy reproduction season. Samples were taken during one designated day from different boats operating in the Gulf of Bejaia (N = 200 fish), and transferred immediately to the laboratory. For each specimen, total weight (TW, g) and gonad weight (GW, g) were measured to the nearest 0.1 g and 0.01 g, respectively. The gonadosomatic index (GSI) was calculated as the ratio of gonad weight to total fish weight and expressed in percent [$GSI = (GW / TW) \times 100$], according to Kume and Joseph (1969). Gonads were collected from each fish, preserved in 10% buffered formalin and stored for histological examination.

Maturity tests were conducted according to a five-point scale (Brown-Peterson *et al.*, 2011): I, immature stage, II, developing stage, III, spawning capable stage, IV, regressing stage, V, regenerating stage.

Histological indices

Testicular samples were processed using standard histological methods: by dehydration in a graded alcohol series, clearing in xylene, embedding in paraffin, sectioning into 3–4 µm thick slices, and then staining with Harris Hematoxylin and Eosin (H&E). The histological sections were photographed with a digital camera at 10× magnification for the image analysis. In the histomorphometric analyses, the number and the area of seminiferous tubules under the 10× magnification were calculated (Fig. 1). The seminiferous tubules number (STN) was counted in a single microscopic field at 10x magnification (corresponding to 484 250 µm²). The determination of the average seminiferous tubules area (STA) was obtained using UTHSCSA Image Tool 2.0 software (University of Texas Health Science Center, San Antonio, TX, USA), twenty-five seminiferous tubules were considered for each fish (Amann, 1982). The 25 tubules were

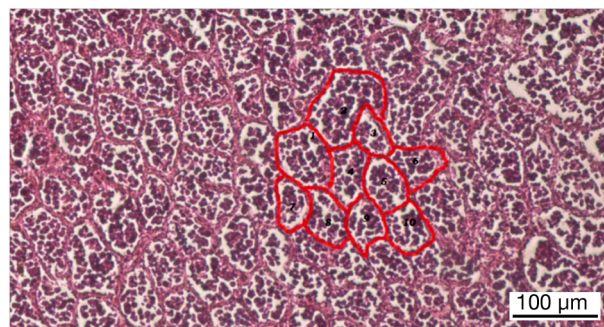


Figure 1. – Photomicrograph of male anchovy testicular tissue illustrating the seminiferous tubules in stage I.

randomly chosen by horizontal scanning, and only those with circular and sub-circular contours were used.

Statistical studies

Statistical analyses were carried out using the R statistical environment version 3.22, a free software environment for statistical computing and graphics. GSI, seminiferous tubule number and seminiferous tubule area, were compared in different sexual maturity stages and months by one-way ANOVA, correlation analysis (between GSI, STA and STN in different sexual maturity stages) and Tukey's honestly significant difference (HSD) as a *post-hoc* test. The HSD test was performed using the "agricolae" package version 1.2-2. Statistical significance was accepted at $p < 0.05$ for all comparisons. Data was presented as mean $\pm$ SEM.

RESULTS

Table I shows the monthly variations of *Engraulis encrasicolus* maturity stages. Stages I and II are evident only from January to April, and stage III is observed only in April. The regressing stage (stage IV) extends from May to October, while the regenerating stage (stage V) lasts from October to January.

Gonadosomatic index (GSI) presents values, which are in accordance with the different maturity stages of the gonads (Fig. 2). GSI increases from the immature (I) to the capable spawning stage (III) and then drops after the regressing stage (IV) and at the regenerating stage (V). GSI presents the lowest values during the immature stage (I) with $0.89 \pm 0.5\%$. The enhancement of GSI at stage II ($2.76 \pm 0.06\%$) and III ($3.87\% \pm 0.11\%$) corresponds to gamete maturation. After the spawning stage (IV), GSI shows a decline, reaching $1.08\% \pm 0.02\%$ corresponding to the regenerating stage (V). GSI reveals significant differences among all the matu-

rity stages except between stages III and IV, representing the spawning capable and the regressing stages. Indeed, there is a significant positive correlation between GSI and stages III and IV ($r = 0.7162$, $p = 0.05$).

Figure 3 represents STN and STA according to maturity stages. The two parameters evolve inversely and present an important negative correlation between STN and maturity stages ($r = -0.67$, $p < 0.001$), and a significant positive correlation between STA and maturity stages ($r = 0.66$, $p < 0.01$), except between stages IV and V. STN (Fig. 3A) and STA (Fig. 3B) express quantitatively the different maturity stages in accordance with GSI (Fig. 2), and thus appear like relevant indicators of reproduction activity. Moreover, these two parameters exhibit a negative correlation with each other ($r = -0.88$, $p < 0.001$) and STN (Fig. 3A) and GSI (Fig. 2) evolve similarly ($r = 0.68$, $p < 0.001$), while STA (Fig. 3B) and GSI (Fig. 2) evolve inversely ($r = -0.61$, $p < 0.01$).

During the research analysis, GSI (Fig. 4) regularly decreased from October to January and then increased to reach the maximum values in May. Figure 5 represents the evolution of STN and STA from October to May. When

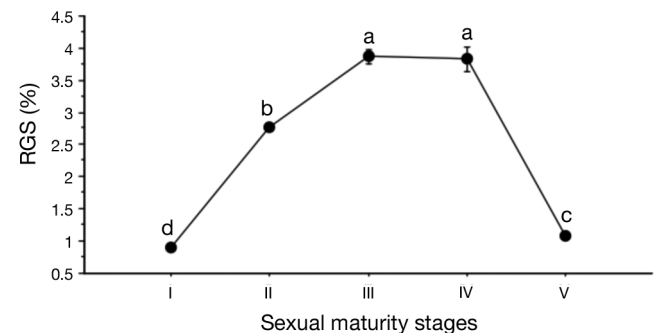


Figure 2. – Mean values of Gonadosomatic Index percentage (GSI) in *Engraulis encrasicolus* male at different gonadal maturity stages (I to V). Data are represented as Mean $\pm$ SEM. Different letters indicate significant differences (ANOVA with Tukey's *post-hoc* test, $p < 0.05$).

Table I. – The annual percentage and number of sampled fish at different maturity stages of male *E. encrasicolus* during the study period (October to May) in the Gulf of Bejaia (n = number of sampled fish)

	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Total fish sampled per stage
I	–	–	–	48.28% (n = 14)	41.38% (n = 12)	10.34% (n = 3)	–	–	29
II	–	–	–	–	20% (n = 6)	40% (n = 12)	40% (n = 12)	–	30
III	–	–	–	–	–	–	100% (n = 15)	–	15
IV	25% (n = 3)	–	–	–	–	–	–	75% (n = 9)	12
V	06.14% (n = 7)	38.60% (n = 44)	43.86% (n = 50)	11.40% (n = 13)	–	–	–	–	114
Total sampled fish per month	n = 10	n = 44	n = 50	n = 27	n = 18	n = 15	n = 27	n = 9	N = 200

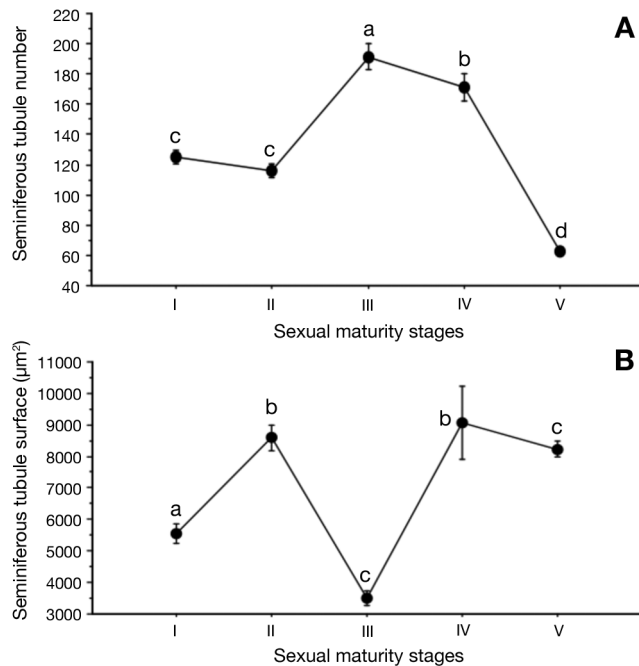


Figure 3. – Mean values of STM and STA in *Engraulis encrasicolus* according to the gonad maturity stages (I to V). **A:** Seminiferous tubules number (counted under 10x magnification). **B:** Seminiferous tubules area (mm^2) (measured on the basis of 25 tubules). Data are represented as Mean $\pm$ SEM. Different letters indicate significant differences (ANOVA with Tukey's post-hoc test, $p < 0.05$).

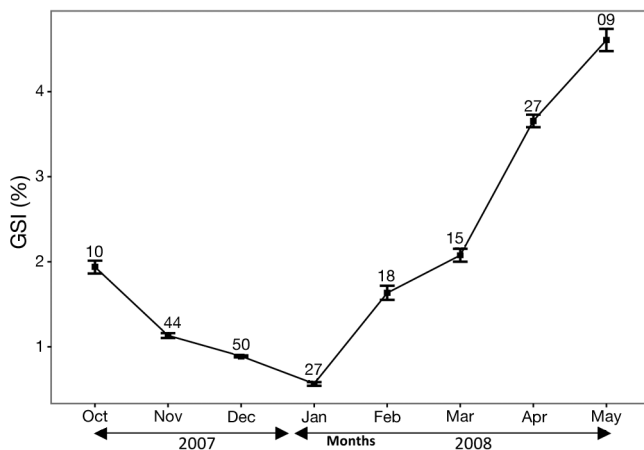


Figure 4. – Mean values of the gonadosomatic index (GSI, represented in percentage) in *Engraulis encrasicolus* male collected in the Gulf of Bejaia from October 2007 to May 2008. Data are represented as Mean $\pm$ SEM. (values indicates the number of fishes).

compared to GSI (Fig. 4), STN (Fig. 5A) evolved similarly except between October and December. A similar evolution was also observed between STA (Fig. 5B) and GSI (Fig. 4), except between March and May, where the two parameters evolve inversely. Compared to STN, STA presents a similar evolution from January to March. The annual change in cell types evolved from October to May. Indeed, from October

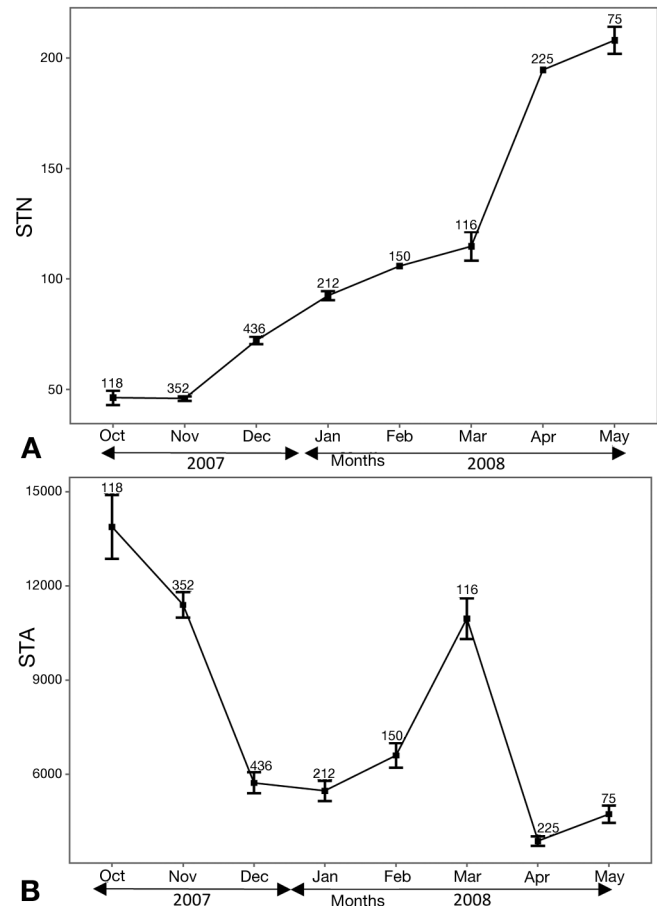


Figure 5. – Mean values of STM and STA in *Engraulis encrasicolus* from October 2007 to May 2008. **A:** Seminiferous tubules number (counted under 10x magnification). **B:** Seminiferous tubules area (mm^2) (measured on the basis of 25 tubules). Data are represented as Mean $\pm$ SEM (values indicate the monthly average number of repetitions).

to December, the testis was mainly composed of spermatogonia (Fig. 6E). In January, spermatocytes started to appear (Fig. 6A) and in February, spermatids also appeared (Fig. 6B). Spermatozoa appeared in April in addition to the presence of spermatids (Fig. 6C), and in May, the testis was full of spermatozoa (Fig. 6D).

DISCUSSION

As ever evoked in the Introduction, when using GSI and the specific validated maturity stages as indicators of the fish spawning period (Berté *et al.*, 2008; Amtyaz *et al.*, 2013), insufficient details and discrepancies are observed compared to the microscopic investigation (Vacchi *et al.*, 2007; Williams, 2007; Costa, 2009). In fact, critics question the use of GSI (De Vlaming *et al.*, 1982; Raubenheimer, 1995; Packard and Boardman, 1999; Smith, 1999) and suggest that GSI is

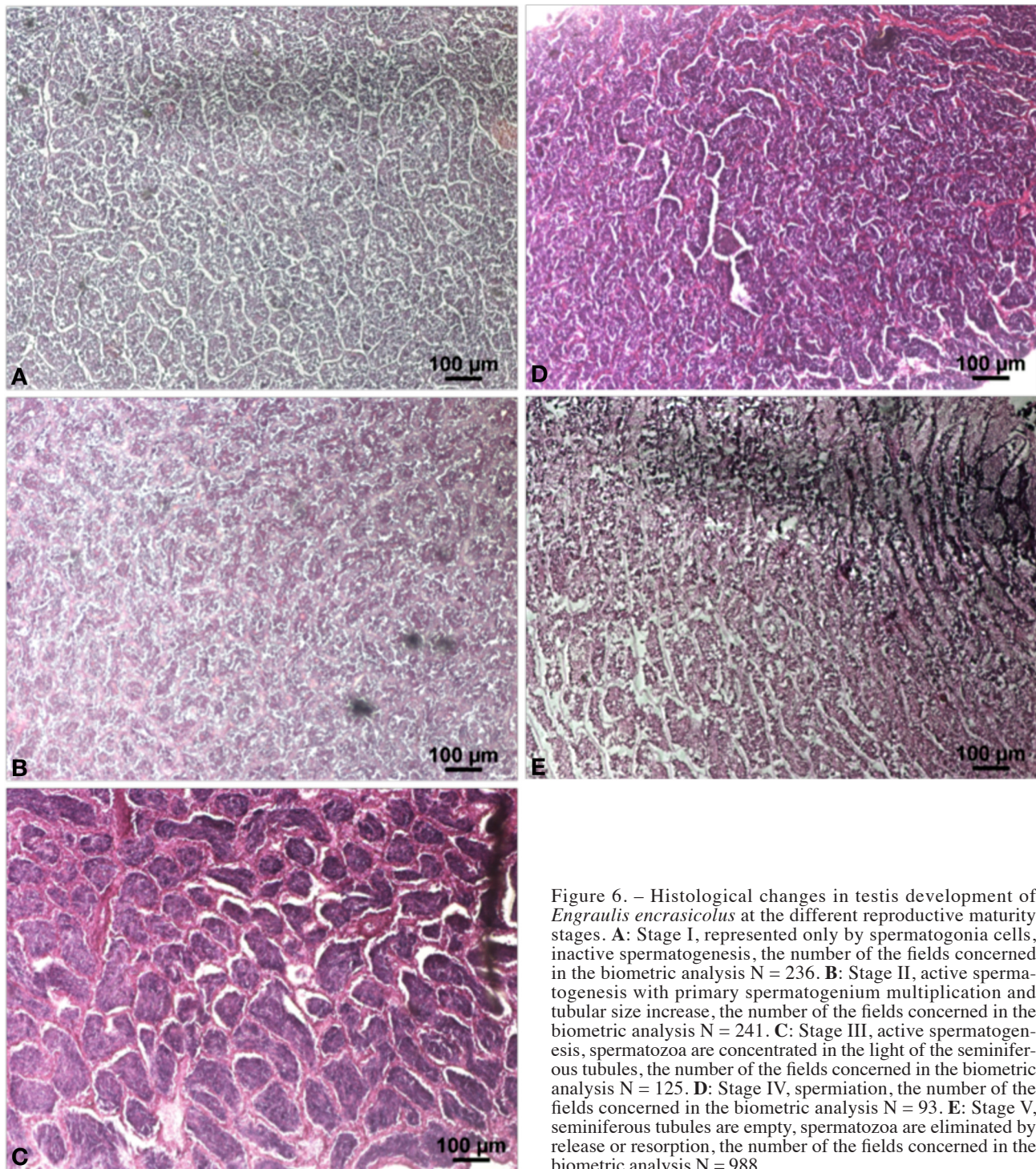


Figure 6. – Histological changes in testis development of *Engraulis encrasicolus* at the different reproductive maturity stages. **A:** Stage I, represented only by spermatogonia cells, inactive spermatogenesis, the number of the fields concerned in the biometric analysis $N = 236$. **B:** Stage II, active spermatogenesis with primary spermatogonium multiplication and tubular size increase, the number of the fields concerned in the biometric analysis $N = 241$. **C:** Stage III, active spermatogenesis, spermatozoa are concentrated in the light of the seminiferous tubules, the number of the fields concerned in the biometric analysis $N = 125$. **D:** Stage IV, spermiation, the number of the fields concerned in the biometric analysis $N = 93$. **E:** Stage V, seminiferous tubules are empty, spermatozoa are eliminated by release or resorption, the number of the fields concerned in the biometric analysis $N = 988$.

not accurate for classification of maturity or reproductive activity, if not calibrated through the use of histology; for instance, it introduces important biases into the data (Packard and Boardman, 1999). The histology generates various information, and offer an accurate record of gonad activity (Hunter and Macewicz, 1985; Blazer, 2002; Brown-Peterson

et al., 2011). Thus, histology is commonly used to validate macroscopic observations and maturity stages (Vitale *et al.*, 2006; Lowerre-Barbieri *et al.*, 2011). However, histology is suggested to be more effective when biometrics approaches are adopted with the measurement of objective parameters (Tomkiewicz *et al.*, 2011).

In that frame, we develop a quantitative histological approach to study male anchovy reproduction activity. Our results revealed a relationship between the seminiferous tubules parameters (STN and STA) and the conventional reproductive indexes (GSI and maturity stages). The correlation analysis demonstrated the existence of an important negative correlation (very strong) and a strong significant positive correlation between maturity stages and STN and STA, respectively. Also, when comparing STN and STA to GSI, a very strong significant positive correlation was found with the first parameter, and a strong significant negative correlation with the second. The lowest values of STN and STA were observed in stage I, reflecting the absence of spermatogenic activity (Hemida, 1988) (Fig. 6A) and the presence of spermatogonia cells only. At this stage (I), the tubule walls are so thick (Bucholtz *et al.*, 2008) that seminiferous tubules occupy less space, resulting in low STN and STA. Between stages I and II, GSI and STA presented similar enhancement, indicating an early development stage with an intense mitotic activity followed by the first meiotic division (Grier, 1992, 1993; Grier and Lo Nostro, 2000) (Fig. 6B).

Stage III was characterised by maximum values of STN and lowest values of STA; this indicates a final spermiogenesis stage where spermatozoa are reaching the final maturation (Fig. 6C). In male, at this stage (III), cells are at their minimal size (Billard, 1979; Barnabé, 1991), causing consequently a shrinkage of seminiferous tubules area (STA) and increased of the seminiferous number (STN) within the microscopic field (Fig. 6C).

Between stage III and stage IV, GSI presented constant values while STN showed a slight decrease. The spawning period is engaged at stage III, represented microscopically by ruptured cysts and semen release in the lumen inducing the low values of STN. At stage IV, STA increased exponentially in relation to the tubule walls fusion (Fig. 6D), creating anastomoses between adjacent lobules (Grier and Taylor, 1998) and to the hydration of the gonads (Legendre and Jalabert, 1988). At stage V, corresponding to the post-reproductive stage, GSI decreased considerably to reach values similar to those observed at stage I; at a microscopic level, this corresponds to low STN and STA values (Fig. 6E).

Generally, in teleost fish, the distinction between immature males and those in regenerating phases are based on histological observation. The criteria to identify an immature testis are the presence of both primary germ cells and spermatogonia (Landry and McQuinn, 1988; Dziewulska and Domagala, 2003; Bucholtz *et al.*, 2008; Brown-Peterson *et al.*, 2011; Follesa *et al.*, 2011; Lawson, 2011). The regenerating phase is distinguished by the presence of spermatogonia and residual sperm in some tubules (Blazer, 2002; Santos *et al.*, 2006; Bucholtz *et al.*, 2008; Brown-Peterson *et al.*, 2011; Lawson, 2011).

When working only with GSI, similar values were observed at stage I and stage V, which avoids to distinguish the two stages. In contrast, STN and STA differ between these two stages, allowing distinction between an immature and a regenerating stage. In fact, the difference between stage I and stage V is only clear by histological observations. When compared to the SMI (spermatogenic maturity index), which is a method based on somatic and germ cell quantification (Tomkiewicz *et al.*, 2011), STN and STA determination is readily achievable as no cell identification is needed within the seminiferous tubules. STN and STA are measured using image analysis software, which does not require a skilled person for the histology evaluation.

Finally, both STN and STA are demonstrated to be potent parameters to study quantitatively and continuously the reproduction activity in male anchovy. The experimental design included comparisons of STN and STA to GSI during the study period (from October to May). As expected, GSI showed variations revealing the reproductive activity; however, no accurate information was provided concerning gonads maturity. In contrast, STN and STA expressed the different reproductive stages with more details concerning gamete maturation and release. STN increased during the period of testis development and sperm production and decreased during sperm release and the end of the spawning season. STA increased from January to March, corresponding to spermatogenesis activity, and decreased between March and May, corresponding to sperm release. From June to September, no sampling was performed because of the absence of commercial catches.

In conclusion, the number (STN) and the area (STA) of the seminiferous tubules should be used as indicators in the investigation of *Engraulis encrasicolus* reproduction activity and probably for other fish. The present method is useful since rapid and routine use are recommended as a standard technique, particularly in multicentre studies where the investigation could be based only on biometry of histological slides.

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